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Identification and Estimation of Microcystins in Freshwater Mussels

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ABSTRACT Accumulation of microcystins mainly produced by cyanobacteria *Microcystis* was investigated for freshwater mussels and fishes collected from a lake where heavy blooms of *Microcystis* occurred every year. The identification of microcystins was performed by HPLC equipped with a frit FAB mass spectrometer. Microcystins LR and RR were identified in the mussels *Unio douglasiae* and *Anadonta woodiana,* whereas no microcystin was identified by the present method in fishes, such as *Cyprinus carpio, Carassius carassius, and Hypomesius transpacificus. Nat. Toxins 5:31–35, 1997.* © 1997 Wiley-Liss, Inc.

Key Words: microcystin; *Microcystis;* mussel; fish; accumulation; water bloom

Eutrophication of freshwater causes occurrence of water bloom of cyanobacteria in many countries of the world. Serious illness and death have been reported among domestic and wild animals since the last century owing to ingestion of toxins produced by cyanobacteria [Carmichael, 1992]. In Japan, the occurrences of toxic *Microcystis* have been reported since Watanabe and Oishi [1980] proved the lethal toxicities of *Microcystis* naturally growing in several lakes and reservoirs. The peptide toxin named microcystin, produced by *Microcystis,* was chemically determined to be composed of seven amino acids, including novel amino acids with 20 carbons called Adda[3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid]. More than 40 components have been reported up to date [Reinhart et al., 1994]. The differences among the components are mainly due to substitution of two L-amino acids and the degree of methylation on d-MeAsp and Mdha. Modification to Adda was shown to affect the lethal toxicity [Stotts et al., 1993].

Compared with other cyanobacterial toxins, microcystins are widely distributed. Reported species include *M. aeruginosa, M. viridis, Anabaena flos-aquae, Oscillatoria agardhii,* and *Nostoc* sp. [Watanabe et al., 1988; Meriluoto et al., 1989; Namikoshi et al., 1990, 1992]. Seasonal changes in both compositions of *Microcystis* population and the toxins were investigated in Lake Suwa and a pond in Tokyo in Japan [Park et al., 1993; Watanabe et al., 1994]. Though a fate of microcystins was followed in the Lake, only low amounts of microcystins were estimated in the lake water, irrespective of the fact of easy release of the toxin into surrounding water in the case of the decomposition by bacteria [Watanabe et al., 1992]. Therefore, other pathways than direct decomposition of microcystins by bacteria must be taken into consideration, as Eriksson et al. [1989] pointed the possibility of accumulation of microcystin in freshwater mussel experimentally.

The present study was conducted to examine the possibility of the accumulation of microcystins in animals in the Lake where a heavy bloom of *Microcystis* occurs every year.

Mussels and fishes collected in Lake Suwa in July and August in 1992, were dissected and kept at −20°C prior to extraction for chemical analysis. The clean-up procedures were essentially same as described previously [Tsuji et al., 1994]. A sample tissue was homogenized for 5 min in 10 ml of 20% aqueous acetic acid by a Polytron homogenizer (Kinematica GMBH, Luzern), shaken for 10 min to obtain the crude extract, and the crude extract was centrifuged at 24,000*g* for 30 min. The precipitate was further extracted twice. The combined supernatant was filtered through a glass fiber filter (Whatman GF/C, Maidstone, U.K.), and the filtrate was applied to a Sep-pak C18 ODS cartridge (5 g, Waters, Millipore Co., Milford, MA). The cartridge was successively washed with 100 ml of water, 20% MeOH-H_2O (100 ml), and 90% MeOH-H_2O (100 ml). The last eluate containing microcystins was evaporated to dryness, dissolved in MeOH (10 ml), and applied to a Sep-pak silica gel cartridge (2 g, Waters). The cartridge was washed with MeOH (30 ml) and the toxins were eluted with 70% aqueous

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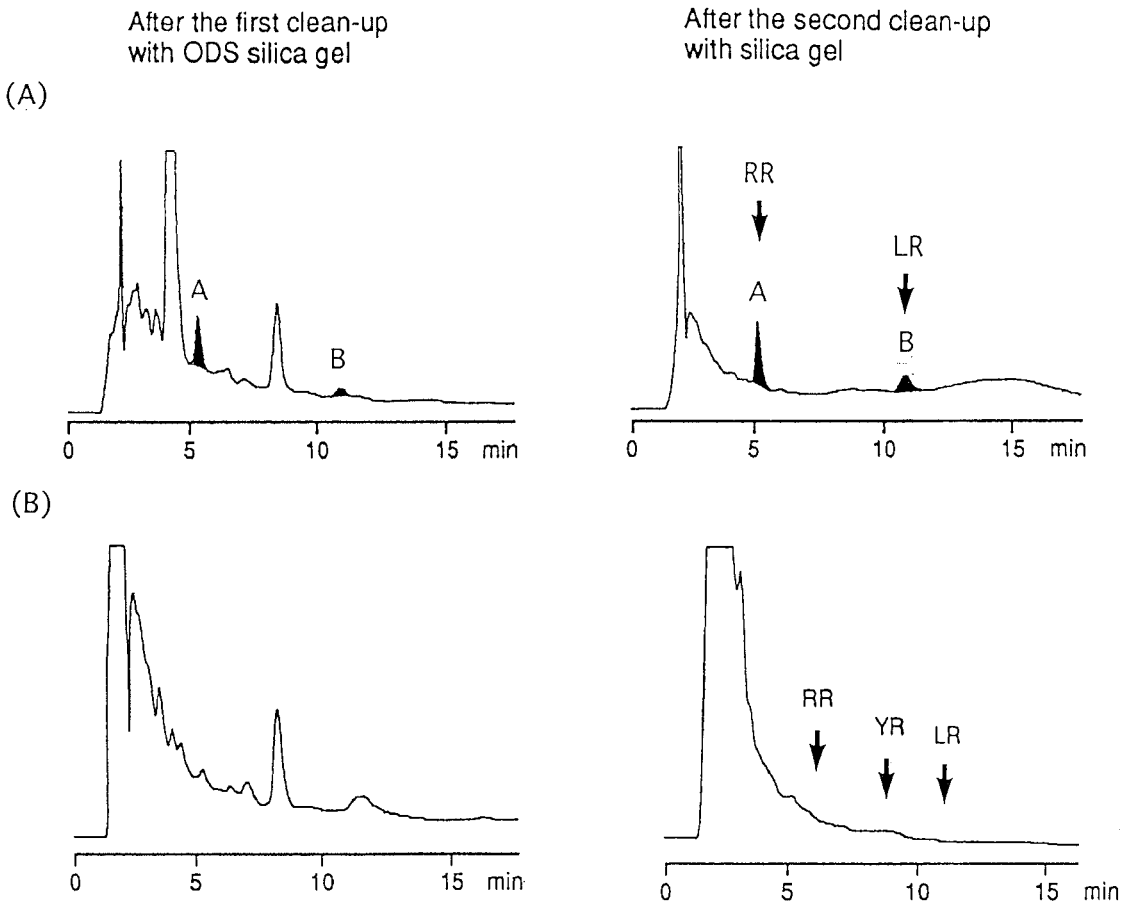

Fig. 1. High performance liquid chromatograms monitored at 238 nm and measured for the hepatopancreases of freshwater mussels after the first clean-up with ODS silica gel (left) and the second clean-up with silica gel (right). **A:** *Unio douglasiae*, **B:** *Cristaria plicata.*

methanol (20 ml). After evaporation of the toxic eluate to dryness, the residue was dissolved in methanol (1 ml) and the solution was used for the HPLC and Frit-FAB LC/MS analyses. Standard microcystins were isolated from cultured stains of *M. aeruginosa* M228 and *M. viridis* TAC 44 and the purity was confirmed by HPLC, TLC and MS [Harada et al., 1988]

High performance liquid chromatography was carried out with an LC-100P HPLC pump (Yokogawa Electric, Tokyo, Japan) on an ODS column (Nucleosil 5C18, 150 × 4.6 mm, Chemco Scientific Co., Osaka, Japan). Sample was separated with mobile phase consisted of MeOH:0.05% TFA (58:42) at a flow rate of 0.8 ml/min. The effluent from the column was monitored with the absorbance at 238 nm using a UV detector.

The flow injection for LC/MS was done with a mobile phase of methanol and 0.05% TFA (56:44) by using an LC-100 P HPLC pump kept at 0.5 ml/min. Develosil ODS-HG column (150 × 0.3 mm, Nomura Chemical Co., Ltd, Seto, Japan) coupled to a mass spectrometer (JMS-AX505W, JOEL Co. Ltd., Tokyo, Japan). The FAB gun was operated at 3 kV with xenon, and the spectrometer was operated at 5 kV accelerating potential.

Clean-up procedure reported previously by Harada et al. [1988] was developed for algal samples and is insufficient for such samples as they contain many more impurities. A modified method developed by Tsuji et al. [1994] enabled detection of microcystins in 1 ng level by using two types of silica gel cartridges for clean-up procedure. However, the method did not give satisfactory results when applied to animal bodies such as mussels and fish. Therefore, in the present study, 20% acetic acid was added to the samples to remove impurities in the first extraction step, and filtration through a glass fiber was performed to remove fine particles which could not be precipitated by a centrifugation at 24,000*g*. Chromatograms monitored for hepatopancreas of two species of mussels were compared after the first clean-up with those after the second clean-up (Fig. 1). In *Unio douglasiae* (A), a small peak expected to be microcystin (Fig. 1A), changed to be clear enough for identification after the second clean-up with a silica gel cartridge. Furthermore a peak around 8 min after the first clean-up with an ODS cartridge, which would be indistinguishable from a peak of microcystin YR, could be removed after the treatment of the second silica gel cartridge. In a case of *Cristaria plicata* (B), the peaks due to impurities observed

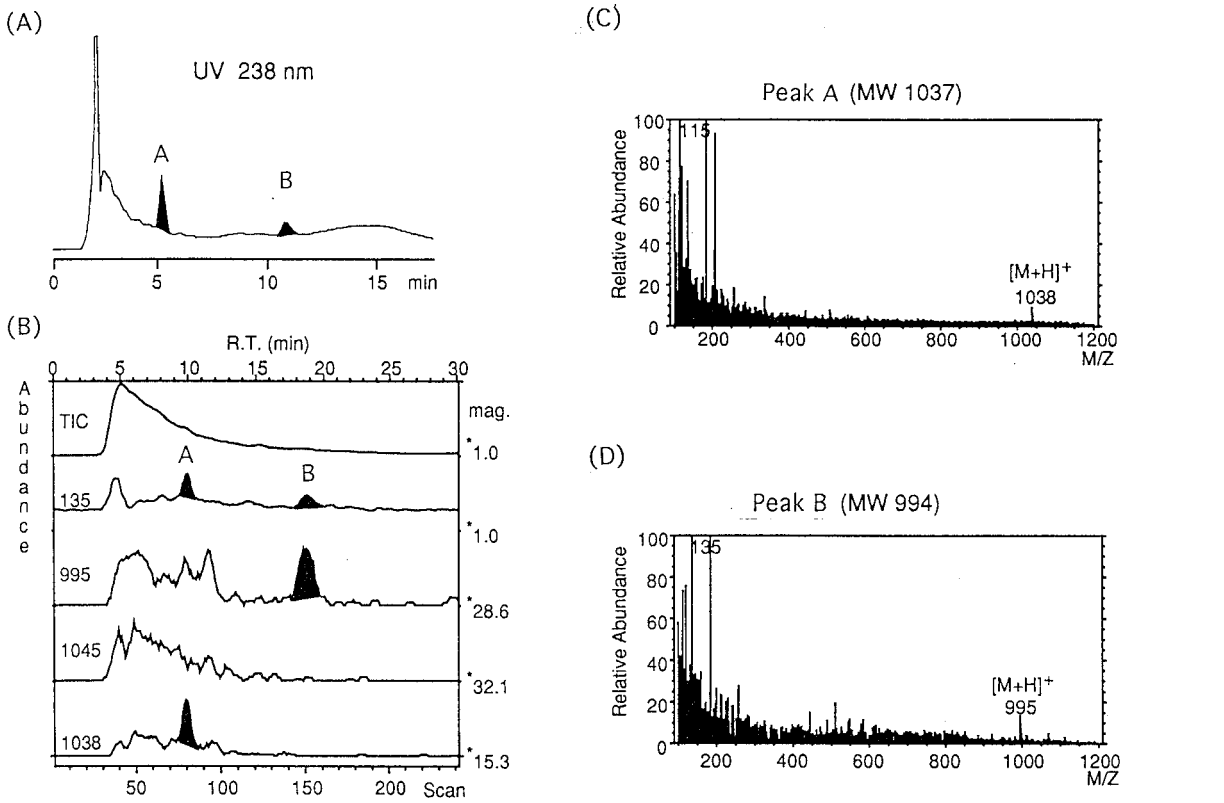

Fig. 2. Chromatograms measured for a hepatopancreas of *Unio douglasiae*. **A:** High performance liquid chromatogram monitored at 238 nm. **B:** Total ion chromatogram and mass chromatograms monitored at m/z 135, 995, 1,045 and 1,038. **C:** Frit-FAB LC/MS mass chromatogram of peak A. **D:** Frit-FAB LC/MS mass chromatogram of peak B.

after the treatment of an ODS cartridge, disappeared after the second clean-up with a silica gel cartridge. A rather flat baseline was observed at the range (5–15 min) where microcystins would be expected. We consider that the sample contained no microcystin. These chromatograms showed that the treatment of two types of the silica cartridge was effective for elimination of coexisting substances other than microcystins.

Identification of microcystins was performed by Frit-FAB LC/MS. Figure 2 shows the results of a chromatogram of HPLC with UV detector at 238 nm [A]; total ion chromatogram and mass chromatograms monitored at m/z 135, 995, and 1045 [B]; and Frit-FAB LC/MS mass spectra of peak A [C] and peak B [D]. They were monitored by using 70% methanol eluate from a silica cartridge, measured on the crude extract of hepatopancreas of *Unio douglasiae*. Adda in microcystin molecules generates characteristic ions useful for identifying microcystins from other compounds [Kondo et al., 1992]. Total ion chromatograms and mass chromatograms monitored at m/z 135 suggested that two compounds corresponding to peaks A and B, respectively, contained Adda. Furthermore, the total ion chromatogram, mass chromatograms monitored at m/z 1,038, and the presence of $[M + H]^+$ ion at m/z 1,038 confirmed that peak A was derived from microcystin RR, which contains two arginines. Similarly, peak B was deduced to be derived from microcystin LR containing leucine (131) and arginine (174), as the peaks were detected by monitoring with m/z 135 and 995, and the mass chromatogram showed $[M + H]^+$ at m/z 995. Chromatograms measured on a hepatopancreas of *Anadonta woodiana* are shown in Figure 3. After the second clean-up with silica gel, the peak A appeared more clearly [B] than the first clean-up with only a ODS silica gel [A]. A total ion chromatogram, mass chromatograms monitored at m/z 135 and 1,038, and the $[M + H]^+$ ion at m/z 1,038 suggested that microcystin RR was present in the sample.

Table I shows the anatomical distribution of microcystins in mussels and fishes. In *U. douglasiae*, the microcystin concentration was the highest in the hepatopancreas followed by gills and muscle. Small amounts of microcystins were also found in the gonads and the alimentary tract. However, in one sample of *U. douglasiae*, no microcystin was detected. Microcystin RR was detected in *A. woodiana*, but not in *Cristaria plicata*. Occurrence of microcystins in three species of fish tested was not confirmed by the present method. Microcystin YR was not detected in any of the samples analyzed.

The present data clearly show that hepatotoxic microcystins were accumulated in freshwater mussels, *U. douglasiae* and *A. woodiana*, which are often dominant species in freshwater environments and occasionally eaten by lakeside people. The accumulation of microcystin was reported by

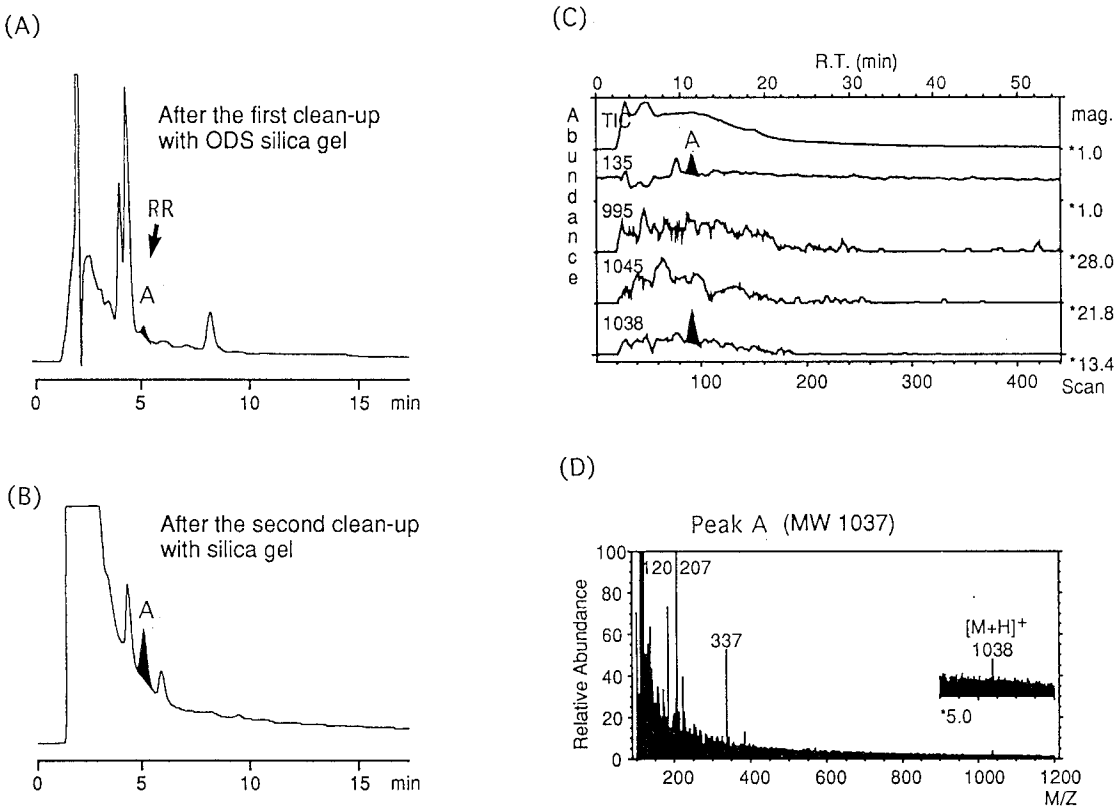

Fig. 3. Chromatograms measured for a hepatopancreas of *Anadonta plicata*. **A:** High performance liquid chromatogram monitored at 238 nm after the first clean-up with ODS silica gel. **B:** HPLC chromatogram after the second clean-up with silica-gel. **C:** Total ion chromatogram and mass chromatograms of peak A monitored at m/z 135, 995, 1,045, and 1,038. **D:** Frit-FAB LC/MS mass chromatogram of peak A.

TABLE I. Microcystin Concentration in Mussels and Fishes

Species	Date	Sample	Weight (g)	Microcystin (μg/sample) RR	Microcystin (μg/sample) LR
Mussels					
Unio douglasiae[a]	July 22, 1992	Hepatopancreas	1.62	2.8	1.6
		Gill and muscle	1.40	1.8	1.0
		Gonad	0.42	0.33	0.17
		Gut	0.42	0.33	0.22
Anadonta woodiana	August 8, 1992	Hepatopancreas	5.35	1.1	ND
Cristaria plicata	July 19, 1992	Hepatopancreas	13.5	ND	ND
Fishes					
Cyprinus carpio	July 23, 1992	Liver	4.0	ND	ND
Carassius carassius	July 23, 1992	Gut	8.5	ND	ND
Hypomesius transpacificus	July 23, 1992	Muscle plus gut	3.5	ND	ND

[a]Data are shown for one sample.

experimentally rearing a freshwater mussel, *A. cygnea*, in cultures of *Oscillatoria agardhii* or by rearing an intertidal mussel, *Mytilus galloprovincitalis*, in cultures of *M. aeruginosa* [Eriksson et al., 1989; Vasconcelos, 1995]. Falconer et al. [1992] reported that an edible mussel, *M. edulis*, collected from brackish water in Australia where heavy water blooms of *Nodularia spumigena* occurred, showed lethal toxicity to mice. However, the authors did not identify and quantify nodularin, a microcystin analogue, in the mussels. Therefore, this is the first study to report the accumulation of microcystins in freshwater mussels collected from natural environment.

A family of microcystin and nodularin was identified also in marine environments. De Silva et al. [1992] reported the presence of the cyclic pentapeptide, motuporin, in the tropical marine sponge *Theonella swinhoei*. Anderson et al. [1993] detected a peak, which was indistinguishable from microcystin LR by HPLC linked with protein phosphatase

assay, in Atlantic salmon infected with netpen disease. They also reported that injection of microcystin LR into salmon caused hystopathological changes in hepatocytes similar to those observed in salmon infected with netpen disease. The report raises an interesting question as to which organism was responsible for the protein phosphatase inhibiting activity. Chen et al. [1993] detected microcystin LR in the mussels from Northwestern Pacific, European, and Canadian coasts by a combination of HPLC and the protein phosphatase inhibiting assay. They estimated a total 3.2 μg hepatotoxin, which was equivalent to 600 ng microcystin LR/g shellfish. Compared with their data, the present study showed a higher microcystin content (6.4 μg) microcystins: 1.7 μg/g shellfish in the case of *U. douglasiae*. Moreover, our mass spectral data provide firm evidence about identification of microcystins.

Dense water blooms of *Microcystis* spp. occur every year in Lake Suwa where the samples were collected [Park et al., 1993]. The concentration of microcystins in surface water was estimated to be in a range from 40 to 100 μg/L and the dominant species was *M. viridis* when mussels and fish were collected (unpublished data). On July 20 in 1992, the first peak was found in cell density of *Microcystis* population as high as 6×10^5/ml. In the lake, eight samples of *Microcystis* cells out of nine collected from the beginning of June to the end of August, showed that amounts of microcystin RR were higher than those of microcystin LR at each sampling period. This feature of microcystin composition seemed to reflect to that in the mussels (Table I). Based on the LD_{50} of microcystins (70–500 μg/kg) [Watanabe et al., 1989; Stotts et al., 1993], about 1.5 μg to 20 μg microcystins can kill a mouse (20 g) by i.p. injection. As shown in Table I, the amounts of microcystins in mussels were higher than 3 μg, which supposedly would kill a mouse by i.p. injection. However, ten times or even a higher amount of microcystins would be needed to kill a mouse by oral administration. The distribution studies with [^{3}H] microcystin LR showed that a high percentage of microcystin remained in the cytosolic fraction of the liver even 6 days after injection, suggesting covalent binding to high-molecular-weight proteins [Robinson et al., 1991a, b]. In the present study, a limited number of mussels and fishes were analyzed at a period of the season of the water bloom of *Microcystis*. Therefore, it is important to assess the potential health risk due to continuous intake of sublethal doses of microcystins present in mussels by increasing the number of both individuals and by extending the observation period.

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